Scientia Agricola
http://dx.doi.org/10.1590/0103-9016-2013-0210

Identification of the *Er*1 resistence gene and RNase S-alleles in *Malus prunifolia* var. ringo rootstock

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Edited by: Antonio Costa de Oliveira

Received June 26, 2013
Accepted October 22, 2013

ABSTRACT: Woolly apple aphid (WAA; *Eriosoma lanigerum* Hausm.) is a major insect pest that has significant economic impact on apple growers worldwide. Modern breeding technologies rely on several molecular tools to help breeders select genetic determinants for traits of interest. Consequently, there is a need for specific markers linked to the genes of interest. Apple scions and rootstocks have an additional barrier to the introduction of pest resistance genes due to the presence of self-incompatibility S-RNase alleles. The genetic characterization and early identification of these alleles can amplify the contribution of a breeding program to the selection of resistant genitors that are as compatible as possible. In this study, we identified the *Er*1 gene involved in the resistance to WAA in *Malus prunifolia* var. ringo, also known as 'Maruba Kaido' rootstock, and we analyzed the inheritance pattern of the WAA resistance *Er*1 gene in a segregant population derived from *Malus pumila* 'M.9' and 'Maruba Kaido' rootstocks. The self-incompatibility of S-RNase alleles S_6S_{26} of 'Maruba Kaido' were also identified along with their inheritance pattern. We also confirmed the identification of the S_1S_3 alleles in the 'M.9' rootstock. To the best of our knowledge, this is the first study to characterize WAA resistance and RNase S-alleles in 'Maruba Kaido'. Furthermore, we discuss the potential use of the genetic markers for these genes and their potential impact on apple breeding programs.

Keywords: Malus pumila, woolly apple aphid, self-incompatibility, bulk segregant analysis, marker-assisted selection

Introduction

Current breeding technologies have included a wide range of traits or selection strategies to satisfy the needs of growers and markets worldwide in developing pest and disease resistant varieties, as well as varieties that meet fruit quality, precocity and yield standards (Evans et al., 2010).

Over the past 25 years, Brazilian apple trees have been grafted mainly onto the 'M.9' (*Malus pumila*) rootstock because of its dwarfing ability. However, 'M.9' rootstock is highly susceptible to pests and diseases (i.e. woolly apple aphid, *Rosellinia* sp and fireblight) (Denardi, 2002).

Woolly apple aphid (WAA; *Eriosoma lanigerum* Hausm.) is a widespread apple pest that can be economically damaging to the Southern Hemisphere (Sandanayaka et al., 2005). Three major WAA resistant genes have been identified—*Er*1 (Knight et al., 1962; King et al., 1991), *Er*2 (King et al., 1991), and *Er*3 (Sandanayaka et al., 2003), which are carried by the apple cultivars 'Northern Spy' (*Malus domestica*), 'Robusta 5' (*Malus* × *robusta*), and 'Aotea' (*Malus sieboldii*), respectively.

Many breeding efforts over the last few years have focused on developing techniques of marker-assisted selection (MAS) that indirectly select genotypes for one or more traits of interest. Although improving the efficiency of selection by making it a more precise system, the technique relies on the identification of a sufficient number of markers. Further complicating breeding practices among apple species is the fact that most apple species exhibit a gametophytic, self-incompatibility (SI) mechanism, which prevents fertilization following self-pollination. The simple genetics of the gametophytic SI system imply that the cross-pollination pattern of an apple variety may be predicted from its RNase S-allele constitution (Broothaerts, 2003).

Considering the paucity of genetic information available about the WAA resistance source 'Maruba Kaido' and its SI systems, this study aimed to identify S-RNase alleles and a WAA resistant gene present in this variety by using a segregant population derived from a 'M.9' × 'Maruba Kaido'. In addition, we also confirmed the identification of the S_1S_3 alleles in the 'M.9' rootstock used by Brazilian breeders.

Materials and Methods

Plant material and phenotypic analysis for resistance to WAA

A segregant population derived from the controlled cross between 'M.9' (*Malus pumila*) and 'Maruba Kaido' (*Malus prunifolia*) apple rootstocks was developed in order to investigate WAA resistance and the self-incompatibility alleles present in 'Maruba Kaido'. The cross was performed in the spring of 2002 in São Joaquim, Santa Catarina, Brazil. The population was composed of 49 F_1 individuals using 'Maruba Kaido' as the pollen donor. The seedlings obtained were planted in pots and maintained in a greenhouse. After one year, the seedlings were transferred to the field in Caçador, Santa

Catarina, Brazil (26° 46' S; 51° 00' W), where they were multiplied using the stool layering technique.

In the following years the progeny was repeatedly inoculated with WAA by attaching heavily infested root pieces to each seedling in the field (Cummins and Aldwinckle, 1983). WAA samples were randomly collected from São Joaquim orchards and colonies were maintained on susceptible plant material. There was no evidence of biotypes being present in these inoculums that could overcome the WAA resistance under investigation. WAA infestations were assessed at various intervals, from 2 to 3 months after inoculation up to four years after transplanting to the field.

The average number of shoots infested per seedling during the evaluations was used in order to classify the susceptibility level using a three point scale adapted from Bus et al. (2008): Resistant = score 1, no infestation; Medium infestation = score 2, up to 2 shoots infested; and Susceptible = score 3, more than 2 shoots infested; scores of 2 and 3 were considered susceptible. Overwintering colonies of WAA are usually found in old pruning scars and only a few survivors were recorded on shoot galls from the previous year's infestation. Therefore, shoots infested in the previous year do not count in the scoring in the following year. Statistical analyses were based on the score of resistance. The χ^2 test was applied to determine the inheritance of the genetic resistance derived from 'Maruba Kaido'.

Bulk segregant analysis using molecular markers linked to the resistance genes *Er*1, *Er*2 and *Er*3

Resistant and susceptible bulks were composed of extreme phenotypes (Resistant = score 1; Susceptible = score 3) based on the phenotypic characterization of the WAA resistance in the 'M.9' × 'Maruba Kaido' population. Bulks were analyzed according to the Bulk Segregant Analysis strategy (BSA) (Michelmore et al., 1991). Each bulk was composed of equal amounts of DNA from 10 genotypes. The resistant and susceptible bulks, parental and positive controls were genotyped with molecular markers (sequence characterized amplified regions – SCARs; simple sequence repeat – SSR; and single nucleotide polymorphism - SNPs) (Table 1), previously described by Bus et al. (2008) as being linked to the *Er*1, *Er*2 and *Er*3 WAA resistant genes.

The SNP NZsn_O05 marker can amplify both *Er*1 and *Er*3 genes. In order to be able to discriminate *Er*1 and *Er*3 genes, we have applied two additional *Er*1 exclusive markers. Samples of the varieties 'Northern Spy' (*M.* × *domestica*), *M.* × *robusta* and *M. sieboldii* accessions collected at the Apple Germplasm Bank (Caçador, Santa Catarina, Brazil) were used as positive controls as they have been previously characterized as presenting *Er*1, *Er*2 and *Er*3 genes, respectively (Sandanayaka et al., 2003). Genomic DNA was extracted from young healthy leaves following the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) and quantified by spectrophotometer. Samples were stored at -20 °C until use. Polymerase chain reaction (PCR) conditions were put in place following Bus et al. (2008). PCR products were visualized by using 1.5 % and 3 % agarose gel electrophoresis and a fluorescent staining.

Validation of WAA resistance gene marker in 'M.9' × 'Maruba Kaido' progeny

The 49 F_1 individuals from the 'M.9' × 'Maruba Kaido' population were randomly screened in a double-blind test coupling phenotypic evaluation with the results of genotype testing to validate the suitability of the NZsn_O05 marker for the *Er*1 gene. At least three replicates of PCR analysis were conducted.

S-locus genotyping

The same DNA samples for the WAA investigation were also used to examine the self-incompatibility RNase alleles present in this population. Sixteen RNase S-allele primers from various sources (Table 1) were used to screen the 'M.9' × 'Maruba Kaido' population. PCR, enzymatic digestion and gel electrophoresis conditions for the S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{16}, S_{19}, S_{20}, S_{22}, S_{23}, S_{24} and S_{26} were performed according to Albuquerque-Junior et al. (2011) with modifications. Small amplified fragments (< 300-bp) were separated on a 4 % denaturing polyacrylamide gel and visualized using silver-staining as described by Creste et al. (2001).

Product sizes were estimated by comparison with a 10-bp DNA ladder. Several scion cultivars of *M. domestica* (Apple Germplasm Bank) were used as positive (S-allele present) and negative (S-allele absent) controls. These cultivars were characterized by Albuquerque-Junior et al. (2011). The χ^2 test was applied to determine the inheritance of the S-alleles derived from 'Maruba Kaido'.

Results

Phenotype and Genetics of WAA resistance

The 'M.9' × 'Maruba Kaido' population had a phenotypic bimodal segregation pattern for the *Er*1 gene. Out of 49 individuals, 29 were classified as score 1 (Resistant - R), 7 as score 2 (Medium infestation) and 13 as score 3 (Susceptible - S) (Figure 1). These results do not differ from the 1:1 (R:S) ratio expected for major genes ($\chi^2 = 1.653$; $p = 0.19$, $df = 1$). Resistant individuals were also checked for root infestation (data not shown).

***Er* resistance gene identification**

The phenotypic characterization revealed that 'Maruba Kaido' WAA resistance is monogenic. Therefore, the BSA strategy was employed to identify the resistance gene (or locus) in the apple rootstock genome. To screen the bulks, four molecular markers previously described as being associated with WAA resistance were used. In the variety 'Northern Spy', the SCAR markers NZsc_GS327 and NZsc_O05 are linked to the *Er*1 resistance gene. The SNP marker NZsn_O05 is linked to the *Er*1 and *Er*3

Table 1 – The sequences and cultivar references of the PCR primers for the sequence characterized amplified region (SCAR), single nucleotide polymorphism (SNP), and simple sequence repeat (SSR) markers linked to woolly apple aphid (WAA; *Eriosoma lanigerum*) resistant genes used in this study. S-RNase specific primers used to identify the self-incompatibility alleles present in *Malus prunifolia* 'Maruba Kaido' apple rootstock are also included.

Target gene	Marker type	Marker name	Product Size (bp)	Cultivar reference	References
Er2	SSR	NZms_EB145764	198	'Robusta 5' (M. ×robusta)	Bus et al. (2008)
Er1/Er3	SNP	NZsn_O05	880	'Aotea1'(M. sieboldii)	Bus et al. (2008)
Er1	SCAR	NZsc_O05	1700	'Northern Spy' (M. domestica)	Bus et al. (2008)
Er1	SCAR	NZsc_GS327	1600	'Northern Spy' (M. domestica)	Bus et al. (2008)
S1	S-RNase specific	FTC168 FTC169	530	Fuji (S1S9)	Sassa et al. (1996)
S2	S-RNase specific	OWB122 OWB123	449	Golden Delicious (S2S3)	Broothaerts et al. (1995)
S3	S-RNase specific	FTC177 FTC226	500	Golden Delicious (S2S3)	Broothaerts et al. (1995)
S4	S-RNase specific	FCT5 OWB249	274	Gloster (S4S19)	Nerum et al. (2001)
S5	S-RNase specific	FTC10 FTC11	346	Gala (S2S5)	Janssens et al. (1995)
S6	S-RNase specific	FTC141 FTC142	850	Not used	Albuquerque Junior et al. (2011)
S7	S-RNase specific	FTC143 FTC144	302	Idared (S3S7)	Janssens et al. (1995); Kitahara et al. (2000)
S9	S-RNase specific	OWB154 OWB155	343	Fuji (S1S9)	Janssens et al. (1995); Sassa et al. (1996)
S10	S-RNase specific	FTC12 FTC228	209	McIntosh (S10S25)	Richman et al. (1997); Kitahara and Matsumoto (2002); Nerum et al. (2001)
S16	S-RNase specific	FTC5 OWB249	274	Baskatong (S16S26)	Verdoodt et al. (1998)
S19	S-RNase specific	FTC229 FTC230	304	Delicious (S9S19)	Matsumoto and Kitahara (2000); Okuno (2000)
S20	S-RNase specific	FTC141 FTC142	920	Mutsu (S2S3S20)	Matsumoto et al. (1999)
S22	S-RNase specific	FTC5 OWB249	274	Alkmene (S5S22)	Nerum et al. (2001)
S23	S-RNase specific	FTC222 FTC224	237	Granny Smith (S3S23)	Schneider et al. (2001)
S24	S-RNase specific	FTC231 FTC232	580	Braeburn (S9S24)	Kitahara et al. (2000); Verdoodt et al. (1998)
S26	S-RNase specific	FCT14 FTC9	194	Baskatong (S16S26)	Verdoodt et al. (1998)

genes in 'Aotea 1'. Furthermore, the SSR marker NZms_EB145764 is associated with the *Er*2 gene in 'Robusta 5'. Expected sizes for each of the four fragments were observed in the controls utilized in this study (Table 1).

The markers NZsc_GS327, NZsc_O05 and NZms_EB145764 revealed the amplification of fragments for both parents and bulks. However, the marker NZsn_O05 (linked to *Er*1 *and Er*3) was polymorphic and only amplified the expected fragment (880bp) in the resistant genitor 'Maruba Kaido' and the resistant bulk (Figure 2).

Both NZsc_GS327 (*Er*1) and NZsc_O05 (*Er*1) amplified a fragment in susceptible parents and progenies that are different from the one associated with resistance. However, it is difficult to differentiate susceptible and resistant alleles because of the similar size and the resistant progeny may also present the susceptible allele. The SSR marker NZms_EB14564 (*Er*2) is also expected to show amplification in 'M.9'. Moreover, NZsnO05 (*Er*1 and *Er*3) shows a complex set of alleles (Bus et al., 2008). With this in mind, we conducted another set of PCR reactions in order to detect the alleles in all four markers in a higher resolution gel (3 % agarose). From this analysis we were able to detect alleles in the NZsc_O05 (*Er*1) and NZsn_O05 (*Er*1 and *Er*3) markers that were present only in the resistant parent individual and the resistant bulk (Figure 3). This evidence suggests that *Er*1 is present and not *Er*3,

since we were able to detect the co-dominant bands in NZsc_O05 and the inheritance experiment showed monogenic resistance. Nevertheless, NZsn_O05 amplifies both genes. To the best of our knowledge, this is the first time that the *Er*1 gene has been described for 'Maruba Kaido'.

Validation of the NZsn_O05 marker

We used the segregant progeny from the cross 'M.9' × 'Maruba Kaido' to validate the link between marker NZsn_O05 and the *Er*1 gene which is present in the resistant parental 'Maruba Kaido'. Fourty nine seedlings were tested for the presence of this marker and the resistant phenotype in a double-blind analysis. Twenty one out of 29 seedlings that were resistant to WAA (phenotype classified as score 1, Resistant) were correctly predicted (Table 2). Therefore, eight plants with the resistant phenotype did not show the *Er*1 gene through PCR analysis. All 20 individuals identified as a susceptible phenotype (score 3) were correctly predicted by the absence of PCR fragment amplification of the NZsn_O05 marker.

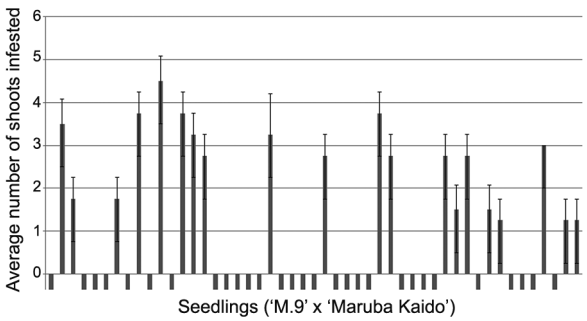

Figure 1 – Average number of shoots infested with WAA in the 49 *Malus pumila* 'M.9' × *Malus prunifolia* 'Maruba Kaido' seedling population during 2003-2006 grown in southern Brazil. Bars under zero indicate resistant individuals. Note: p (χ^2 1.653; df 1) = 0.19.

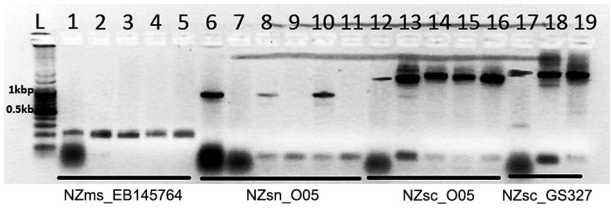

Figure 2 – SSR, SNP and SCAR tagging *Er* genes resistant to *Eriosoma lanigerum* by bulk segregant analysis using primers NZms_EB145764, NZsn_O05, NZsc_O05 and NZms_GS327. Lines: Positive Controls (1 - *Malus* × *robusta*, 6 - *M. sieboldii*, 12 - Northern Spy' (*M.* × *domestica*), 17 - Northern Spy' (*M.* × *domestica*)); negative control (7); resistant parent *M. prunifolia* 'Maruba Kaido' (2, 8, 13, 18); susceptible parent *M. pumila* 'M.9' (3, 9, 14, 19); resistant bulk (4, 10, 15) and susceptible bulk (5, 11, 16). L: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas Molecular Biology Tools). Reference literature for the primers are indicated in Table 1.

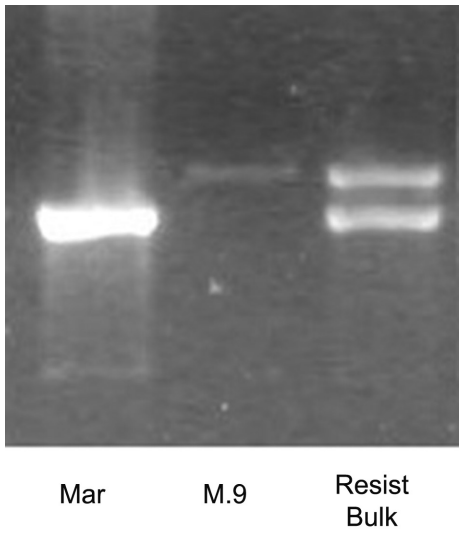

Figure 3 – Amplification of the co-dominant NZsc_O05 marker (Bus et al., 2008) in *Malus prunifolia* 'Maruba Kaido' resistant parent, *M. pumila* 'M.9' susceptible parent and resistant segregant F1 bulk.

Self-incompatibility RNase alleles

RNase S-alleles from apple cultivars have been previously cloned by several authors (Broothaerts et al., 1995; Janssens et al., 1995; Sassa et al., 1996; Verdoodt et al., 1998; Matsumoto et al., 1999; Matsumoto and Kitahara, 2000; Nerum et al., 2001; Schneider et al., 2001; Kitahara and Matsumoto, 2002; Broothaerts, 2003). Based on the nucleotide sequences of the S-alleles encoding S-RNases, a PCR on restriction fragment length polymorphism analysis method (PCR-RFLP) for S-allele identification was used (e.g. Albuquerque-Junior et al., 2011). The method consists of PCR amplification of each S-allele using specific primers for individual S-genes. We used 16 primer combinations to screen the most common apple rootstocks present in Brazilian orchards.

Our results confirm the presence of S_1 and S_3 haplotypes for the 'M.9' cultivar previously detected by Matsumoto et al. (2003). We also identified S_6 and S_{26} in the 'Maruba Kaido' rootstock and we believe that this is the first time that these alleles have been observed and documented for this variety. S_{26} is a rare allele which has been shown to differ from other S-alleles and it has been detected in the crabapple variety 'Baskatong' (*M. domestica* × *niedzwetzkyana)*, *M. floribunda* 821 (Verdoodt et al., 1998; Broothaerts, 2003) and in *M.* × *domestica* old and *M. sylvestris* hybrids (Dreesen et al., 2010).

The segregation pattern for the S-alleles in the 'M.9' × 'Maruba Kaido' population differed significantly from the 1:1:1:1 ratio expected for independent genes (χ^2 = 23.153; p = 0.0001, df = 1) (Table 3). The most frequent S-genotype was S_3S_{26} (48.9 %) followed by S_1S_6 (38.3 %), S_3S_6 (8.5 %) and S_1S_{26} (4.3 %).

Table 2 – Marker-assisted selection validation for Er1 (WAA; *Eriosoma lanigerum*) resistance gene from *Malus prunifolia* 'Maruba Kaido'. The individuals are derived from the segregant progeny between M. pumila 'M.9' and 'Maruba Kaido' apple rootstocks.

	Woolly apple aphid phenotype			Segregation		Total
	Score 1	Score 2	Score 3	Resistant	Susceptible	
Number of seedlings	29	7	13	29	20	49
+	21	0	0	21	0	21
-	8	7	13	8	20	28

Note: Resistant = score 1, no infestation; Medium infestation = score 2, up to 2 shoots infested; Susceptible = score 3, more than 2 shoots infested. "+" indicates amplification of the expected fragment by PCR analysis. "-" indicates no amplification by PCR analysis.

Table 3 – Segregation pattern of S-alleles S_6, S_{26}, S_1 and S_3 from the inter-specific cross between *Malus pumila* 'M.9'and *M. prunifolia* 'Maruba Kaido' apple rootstocks.

Parental		'Maruba Kaido'		
		S6	S26	Total
'M.9'	S1	18	2	20
	S3	4	23	27
	Total	22	25	$\chi 2 = 23.15$

Note: $p = 0.0001$

Discussion

Woolly apple aphid is a pest which can have major economic consequences for apple growers, particularly in Brazil and other countries in the Southern Hemisphere (Denardi, 2002). Despite the fact that resistance of 'Maruba Kaido' to WAA was described more than 30 years ago (Way et al., 1990), the resistance gene has not been utilized in Brazilian commercial apple breeding. The hesitation to include this resistant variety in breeding programs is due to the lack of knowledge of the inheritability of such genes, thus making commercially desirable resistant phenotypes difficult to obtain.

Bulked segregant analysis (Michelmore, 1991) in combination with SCARs markers (Paran and Michelmore, 1993) are powerful techniques for identifying markers tightly linked to, or co-segregating with, genes underlying monogenic traits (Malek et al., 2000). Using these techniques, coupled with phenotypic analysis, we were able to identify a new source of resistance to WAA from the 'Maruba Kaido' rootstock.

The first evidence of a bimodal segregation pattern of WAA resistance was provided by Bus et al. (2008), suggesting the presence of major genes for resistance in 'Northern Spy' (*Er*1), 'Robusta 5' (*Er*2) and 'Aotea 1' (*Er*3). However, significant segregation distortions towards either resistance or susceptibility were observed for all three genes. Resistance in other apple species is monogenic and located in the apple chromosome 8 for the *Er*1 and *Er*3 genes and chromosome 17 for the *Er*2 gene (Bus et al., 2008). In addition, the possibility of a potential link between *Er*1 resistance gene and the SI locus was discarded by Tobutt et al. (2000) after investigation with the aid of the Got-1 and Got-4 isozyme markers.

The successful transferability of *Er*1 gene marker to 'Maruba Kaido' rootstock and its validation are of major interest to apple breeders in Brazil. The availability of these molecular markers represents an additional benefit to the breeding program, as it is possible to screen for specific genes. No false positive genotypes were screened for the presence of the *Er*1 gene and therefore the marker combination assures a high degree of certainty that the gene will be present in subsequent progenies.

Our results accurately predicted both resistant genotypes and phenotypes at a rate of 72.41 % (PCR results in correlation to phenotypic characterization). Previous studies predicted 89.6 % of all resistant individuals from the progeny 'Royal Gala' × 'Northern Spy' segregating for the *Er*1 gene (Bus et al., 2008). Although the percentage of correct prediction of *Er*1 was not high (72.41 %) considering the distance of 4 cM from the gene (Bus et al., 2008), the NZsn_O05 marker did not provide false positive results, which are more problematic to breeding programs. The lack of detection of the *Er*1 gene in some of the phenotypically resistant individuals might be related to either an incorrect phenotypic characterization or mismatched amplification of PCR. Moreover, the identification of the *Er*1 in 'Maruba Kaido' brings another genetic resource for developing WAA resistance. By pyramiding multiple genes, this resource can be used to create new rootstock cultivars with durable pest and disease resistance.

We were also able to confirm the S_1S_3 self-incompatibility alleles for 'M.9' and identify the S_6S_{26} alleles in 'Maruba Kaido' for the first time. As expected for an S-compatible cross, the frequency of the four segregating S-alleles was similar ($\chi^2 = 0.852$; $p = 0.64$, $df = 1$); however, there was genotypic segregation distortion towards two types (S_3S_{26} and S_1S_6) in the interspecific progeny. One explanation for segregation distortion is the presence of post-zygotic barriers. Fertile hybrids are often obtained from pollination between *Malus* species with compatible S-alleles. However, hybrid lethality is commonly observed. In this study, approximately 300 seeds were obtained from the interspecific cross, but only 100 were able to germinate and only 60 became established. Of the 60 seedlings, three were triploids and eight were produced through contamination by foreign pollen (data not shown).

Alternatively, sequence similarity of some S-alleles, which would produce a partial incompatibility response, could lead to the segregation distortion. Heng et al. (2011) investigated the recognition speci-

ficity of self-incompatibility in *Pyrus* sp. and *Malus* sp. and observed that RNase S-alleles present in both species have maintained the same recognition specificity after the divergence of the two species. Heng et al. (2011) also observed that amino acid substitutions found between *PpS 8* -RNase and *MsS 3* -RNase do not alter the recognition specificity. Therefore, further studies on the sequence similarity and incompatibility reaction must be performed in order to investigate possible partial incompatibility between parents. Furthermore, S_6 and S_{26} sequences are not yet fully characterized in the literature. Previous studies have determined that mutations in the nucleotide sequence might change amino acid sites that determine specificity differences (Vieira et al., 2010). Because of self-incompatibility, most apple cultivars need cross-pollination in order to bear fruit. Discrimination of S-genotypes of apple cultivars is, therefore, important for selecting the proper pollen donor in fruit production (Kim et al., 2009; Li et al., 2011).

Brazilian apple orchards are in real need of new resistant rootstocks that include the traits of dwarfing and fruit quality. To date, the wide use of 'M.9' and 'M.26' rootstocks has increased production by improving fruit quality and enabling mechanized harvest; however, these rootstocks demand higher amounts of insecticides and fungicides. Therefore, integrating knowledge of the genetic identification of both WAA resistance and self-incompatibility in 'Maruba Kaido' could lead to the development of genetically resistant cultivars for use in traditional breeding.

The use of such cultivars would benefit not only Brazilian and worldwide apple breeders, but consumers as well. Nonetheless, further work should be done in order to characterize the genomic region related to WAA resistance by sequencing the genes already identified and to prospect other alleles. Since the malus genome is now available in public databases, denser genetic maps around these genes could be developed, which will then help the development of other molecular markers with greater potential to improve their effectiveness as marker assisted selection tools in other genetic backgrounds.

Acknowledgments

This research was supported by grants from The Development Support Project for Agricultural Technology in Brazil (Prodetab), The Brazilian National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES). The authors thank J. Bonetti and Albuquerque-Junior for their help with sample collection. We would like to acknowledge financial support provided by CNPq for scholarships granted to RON and SZAT, and by CAPES for a scholarship given to ACMD. The authors also thank the anonymous reviewers and Vincent Bus for their insightful comments and Dr. Evelyn Nimmo for English language editing of the manuscript.

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